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Biochimica et Biophysica Acta 1709 (2005) 195 – 202
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Review

Old and new data, new issues: The mitochondrial $\Delta\Psi$

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Received 2 June 2004; received in revised form 15 July 2005; accepted 26 July 2005
Available online 24 August 2005

Abstract

New and old data pertinent to the electrochemical potentials across the inner mitochondrial membrane are reviewed with the intent of reconciling the various findings in the light of new perspectives provided by more recent knowledge. A careful scrutiny of old data permits ruling out the presence of a significant metabolically dependent electrical membrane potential. Recent technological advances make it possible to test the proposed alternatives. These proposals recast the original idea, and the possible mechanisms that are emerging also invoke a protonmotive force. Our conclusions that $\Delta\Psi$ is not involved in oxidative-phosphorylation finds parallel observations in *Halobacterium halobium* [H. Michel, D. Oesterhelt, Electrochemical proton gradient across the cell membrane of *Halobacterium halobium*: comparison of the light-induced increase with the increase of intracellular adenosine triphosphate under steady-state illumination, Biochemistry 19 (1980) 4615–4619] and thylakoid vesicles [D.R. Ort, R.A. Dilley, N.E. Good, Photophosphorylation as a function of illumination time II. Effects of permeant buffers, Biochim. Biophys. Acta 449 (1976) 108–129] in which light-induced ATP synthesis occurs in the absence of an apparent $\Delta\Psi$ or ΔpH, suggesting the presence of mechanisms similar to the one proposed for mitochondria.

Keywords: Mitochondria; Membrane potential; Bioenergetic; Oxidative-phosphorylation

The past 20 years have produced technical advances that have allowed science to examine new questions as never before and have provided us with an unprecedented amount of information, including some unexpected answers. It is therefore important to examine old questions to see where new approaches may be of value and whether old ideas have withstood the test of time. In bioenergetics, the past 30 years have been dominated by the chemiosmotic model which proved very fruitful and at times has had excellent predictive value. Its success has discouraged examining other possibilities and details. The present discussion addresses itself to these questions as related to mitochondria.

Recent EM tomographic reconstruction of mitochondria under different metabolic states suggests that there may be intramitochondrial compartments not previously suspected [1]. For example, if the intracristal spaces or a fraction of these spaces were closed vesicles, they could accumulate high concentrations of protons. The technology to examine this question is relatively simple (see Section 4, below).

It has become increasingly obvious that H^+ can traverse a path through protonatable groups (such as amino acid side chains) or an organized water environment [2,3] when being transferred through a protein molecule. The data, using site-specific mutagenesis to replace amino acids shows their involvement in proton transfer (e.g., ref. [4]). Likewise, current evidence indicates that protons generated at the surface of a bilayer membrane diffuse laterally [5–7] (through the polar groups at the surface of phospholipids or organized water at the surface), suggesting that localized protons could be directly coupled to the phosphorylation of ADP when the protons are channeled through the ATP-synthase or alternatively exchange with ions at symports or antiports. A role of surface protons in bioenergetics has in fact been proposed from results obtained with the purple membrane of *Halobacterium salinarium* [5,7]. Theoretical considerations [8,9]) and experimental results [10] indicate that a coupling between proton donor and acceptor sites in a bilayer can be direct without involving the bulk phase. The

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doi:10.1016/j.bbabio.2005.07.008

limiting distance between the two has been estimated to be considerable (nanometers or μm depending on buffers).

The chemiosmotic model for the coupling of electron transport to phosphorylation invokes the presence of a protonmotive force constituted by the electrochemical potential of protons. A coupling between proton influx and the synthesis of ATP has also been shown in experiments with intact mitochondria [11] and proton efflux in submitochondrial preparations (which have an inverted polarity) [12].

Our conclusions that $\Delta\Psi$ is not involved in oxidative-phosphorylation finds parallel observations in *Halobacterium halobium* in which light-induced ATP synthesis occurs in the absence of an apparent $\Delta\Psi$ or ΔpH [13].

In thylakoid vesicles, there is a good deal of evidence for a possible role of a pH gradient and $\Delta\Psi$ (see refs. [14,15]). However, photophosphorylation has been shown to occur in the absence of both ΔpH (using permeant buffers) and $\Delta\Psi$ (controlled with K^+ and valinomycin) [16] arguing for a direct role of protons in photophosphorylation without the necessity of gradients in agreement with our proposals for mitochondria.

In mitochondria, the protonmotive force is thought to be constituted by a pH gradient and an electric membrane potential ($\Delta\Psi$) across the mitochondrial inner membrane. The presentation that follows examines the data on which this premise is based and it contends that in mitochondria, there is no metabolically dependent $\Delta\Psi$ and that some other mechanism must be operative. If this conclusion is correct, such an evaluation is important because new outlooks and biotechnological approaches allow us to examine possible alternatives.

It is important to note that these proposals recast the original idea, and the possible mechanisms that are emerging also invoke a protonmotive force. These proposals can be tested with present techniques.

In addition to the discussion presented below, it should also be noted that an electrogenic pump producing a sufficiently high $\Delta\Psi$ would have to exhibit an efflux of 10^{-6} mol H^+/g protein (Mitchell's calculation [17] with which we concur). Measuring this efflux should be easy with present (or past) technology. We are not aware of a demonstration of such an efflux in the absence of ion transport. On the contrary, data have been presented to show that there is no significant H^+ efflux induced by metabolism [18]. In the control [18], the direct delivery of the equivalent H^+ concentration to the outside medium can be readily measured.

The discussion will first examine the original results based on the distribution of K^+ or Rb^+ in the presence of valinomycin (Section 1) and of lipophilic probe molecules (Section 2). These are the techniques that have been use to estimate $\Delta\Psi$ (e.g., see [19]) and the sets of data collected with these approaches have been offered as the basis for concluding that there is a metabolically dependent $\Delta\Psi$. Section 3 will examine the data collected with micro-electrodes using giant mitochondria. Section 4 discusses possible new experimental approaches for questions that have ambiguous answers or are still unanswered.

1. Experiments using the distribution of K^+ in the presence of valinomycin

The claims of a meaningful metabolically dependent $\Delta\Psi$ in experiments using the distribution of K^+ does not withstand careful scrutiny. In the original study of Mitchell and Moyle [20], $\Delta\Psi$ is calculated from the distribution of K^+ across the inner mitochondrial membrane in the presence of valinomycin. This antibiotic increases the permeability of membranes to K^+ and may have other effects (see below). In these experiments, the mitochondria were partially depleted of K^+ and were then energized (by introduction of O_2) in the presence of substrate and at various external K^+ concentrations (ranging from about 0.012 to 10 mM). Presumably, the data provided the values of K^+ needed for the calculation of $\Delta\Psi$.

The values for the $\Delta\Psi$ calculated by Mitchell and Moyle [20] depend entirely on the K^+ concentrations in the outside medium chosen in the experiments and have no relation to metabolism (see below). As discussed below, there are solid reasons for considering the internal mitochondrial concentration of K^+ constant and 150 mM. Assuming other values does not change the argument significantly (see below). With this assumption, the values calculated in the same manner as in the original study using the Nernst equation are approximately the same as those calculated by Mitchell and Moyle [20] (Fig. 1) and previously attributed to the uptake of K^+ driven by the presence of $\Delta\Psi$. The agreement between the two sets of values indicate that the calculated

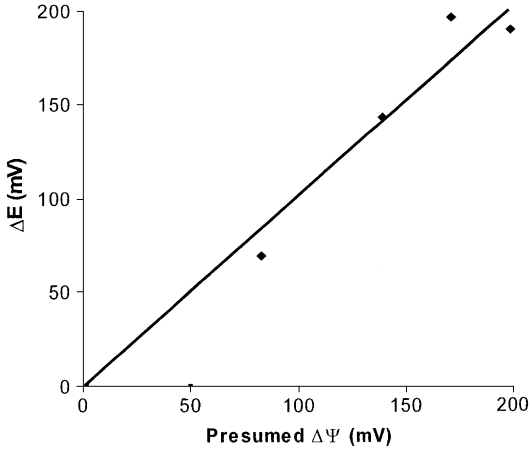

Fig. 1. The calculated $\Delta\Psi$ (Mitchell and Moyle, 1969) results from the external concentration of K^+ and not the uptake of K^+ by mitochondria. Comparison of the presumed $\Delta\Psi$ calculated by Mitchell and Moyle [20] and a diffusion potential (ΔE) calculated with the Nernst equation assuming a constant internal concentration of K^+ (150 mM) and the external concentrations of K^+ [$(K^+)_o$] reported by Mitchell and Moyle [20]; we used the median values of pK_o [$-\log (K^+)_o$] reported in that work. The line represents perfect equivalence between the two sets of values.

$\Delta\Psi$ does not depend on K^+ taken up and any metabolically dependent $\Delta\Psi$, if present, is negligible.

The choice of a constant internal mitochondrial concentration of 150 mM K^+ is not arbitrary. It is based on the following considerations: (a) mitochondria are perfect osmometers (e.g. [21]); K^+ is the highest contributor to the internal osmotic pressure and the internal osmotically active components must match the external concentrations of solutes, approximately 300 mosmolal in the experiments of Mitchell and Moyle [20] and those of Nicholls [22], (discussed below) so that either during the uptake or the exit of K^+, the internal concentration of this cation cannot vary significantly. (b) Rottenberg [23] reports an internal K^+ of 157 mM in 250 mosmolal medium (his Table 1). Corrected for binding of K^+ (in our hands 5.5 mM from his Fig. 2) and adjusted for an external concentration of 300 mosmolal external medium, this becomes 182 mM. In other experiments of the same study, it is approximately 100 mM (his [23] Table V; corresponding to 120 mM at 300 mosmolal concentration of the medium). (c) The constancy of the internal concentration of K^+ as well as the value we chose for the calculations is supported by experimental data [23,24] which invariably find the uptake of K^+ in the presence of valinomycin is matched by an equivalent uptake of water. The swelling of mitochondria produced by various concentrations of valinomycin in metabolizing mitochondria and 250 mosmolal external medium (Fig. 4 of Rottenberg [23]) corresponds to volume change of approximately 9.43×10^{-3} µl per nmol K^+ taken up, equivalent to the ability to maintain an internal concentration of 106 mM (or 127 mM at a 300 mosmolal external concentration) regardless of the degree of swelling. However, the choice of the value 150 mM internal

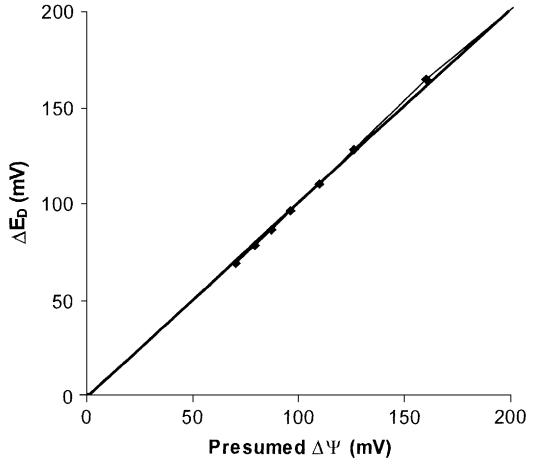

Fig. 2. The electric potential of mitochodria in the presence of K^+ and valinomycin corresponds quantitatively to a diffusion potential (ΔE_D) and not the result of an electrogenic pump. Comparison of the $\Delta\Psi$ calculated by Nicholls [22] from the $^{86}Rb^+$ distribution and the diffusion potential (ΔE_D) calculated using the Nernst equation from the external K^+ [$(K^+)_o$] reported by Nicholls [22] and a constant internal concentration of K^+ of 150 mM. We used the values in Fig. 3B of that author. The heavy line represent perfect equivalence between the two sets of values. The lighter line is a fit of the actual points.

K^+ is not crucial to this argument. The uptake of K^+ for the experiments calculated from the results in the table of Mitchell and Moyle [20] is trivial compared to the contribution of the external concentrations of K^+ even using the same assumptions of those authors (no osmotic behavior, 0.4 g of water/g of protein, no internal K^+ at 0 time). It ranges from 30 to 80 mM, except for item IV in the table (244 mM) which corresponds to the conditions of the experiment of Rottenberg [23] discussed below.

The data of Rottenberg [23] (his Table V) show a constant internal K^+ concentration in experiments with an external concentration of 10 mM KCl in the presence of valinomycin. Regardless of metabolic conditions, he finds the $(K^+)_i/(K^+)_o$ ratio to be between 11 and 10, which would permit a calculated $\Delta\Psi$ of about −60 mV in the presence or absence of metabolism (not far from the value calculated in item IV in the table reported by Mitchell and Moyle [20] of −83 mV at a slightly lower external K^+ concentration). Although metabolism in the presence of valinomycin induce a substantial uptake of K^+, (270 nmol/mg dry weight) compared to mitochondria without an energy source (in the presence of cyanide and oligomycin, 15 nmol/mg dry weight), the ratios of $(K^+)_i/(K^+)_o$ remain exactly the same because of the corresponding change in internal mitochondrial volume.

In experiments similar to those of Mitchell and Moyle [20], Nicholls [22] follows the distribution of $^{86}Rb^+$ at varying external K^+ concentrations in the presence of valinomycin in metabolizing mitochondria. In these experiments the total membrane potential (ΔE_T) would be that imposed by the K^+ diffusion potential (ΔE_D) plus the potential derived from an electrogenic process ($\Delta\Psi$), i.e., $\Delta E_T = \Delta E_D + \Delta\Psi$. These principles are well recognized as illustrated by the electric potential in muscle or nerve after stimulation, which corresponds to the K^+ diffusion potential and in addition an electrogenic potential resulting from the Na^+ pump. In the experiments of Frumento [25], the membrane potential originally poised at the resting potential level is enhanced by the pump. The operation of the electrogenic Na^+ pump is activated by the injection of Na^+ into the muscle fiber.

In the virtual absence of metabolism in the presence of valinomycin and various concentrations of K^+, mitochondria exhibit a diffusion potential as shown by the distribution of a the cationic compound triphenylmethylphosphonium ($TPMP^+$) [26]. The plot (Fig. 2) of the values calculated by Nicholls [22] (using the data shown in his Fig. 3B) against those calculated for the K^+ diffusion potential assuming a constant internal concentration of K^+ (150 mM) shows that the two are almost identical. Again, there is no evidence of the presence of a $\Delta\Psi$. Since the calculated values depend solely on the varying the external concentration of K^+, the results do not reflect a metabolically dependent $\Delta\Psi$.

A similar conclusion can be reached in other experiments, for example, those of Holian and Wilson [26]. In this case, from our limited calculations we can account for most of the reported supposed $\Delta\Psi$ (in this study, estimated from

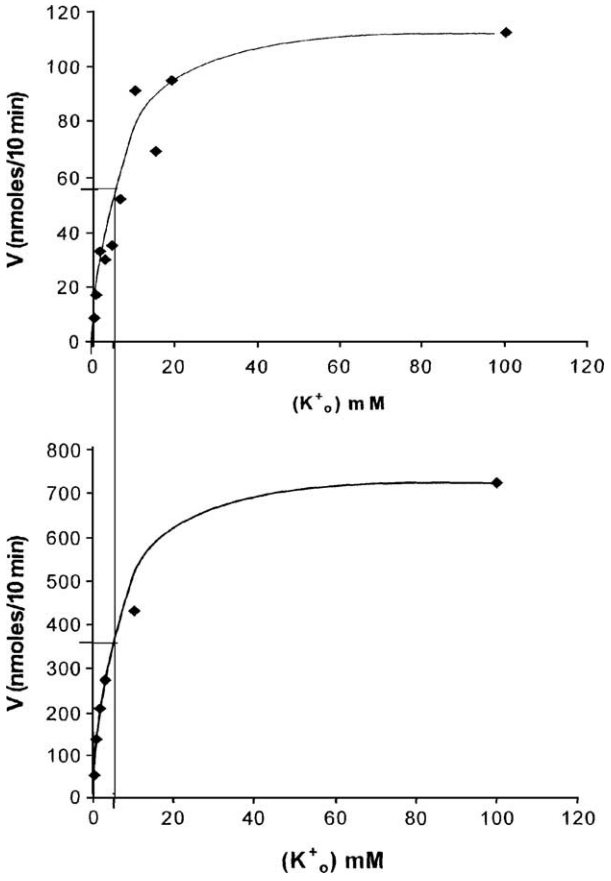

Fig. 3. The uptake of K^+ at varying external K^+ exhibits saturation kinetics and indistinguishable K_ms in the presence or absence of valinomycin. The rate of uptake of K^+ (nmol/10 min/mg dry weight) as a function of medium K^+ concentration calculated from Fig. 5 of Rottenberg [23]. The top part: no valinomycin; the bottom part: valinomycin (0.15 μg/ml) added. The lines represent Michaelis–Menten kinetics. The vertical line has been drawn for a value of K_m of ~5 mM.

the distribution of $TPMP^+$) in the presence of metabolism and valinomycin by a diffusion potential, mostly dependent on the external K^+. Our calculated values (assuming a constant internal mitochondrial concentration of K^+ of 150 mM) are only approximately 20% smaller than those calculated by the authors (we calculate 128, 96 and 82 mV where they reported corresponding $\Delta\Psi$ values of 150, 122 and 102 mV; their Table 1, part B).

Some of the results of Nicholls [22] supposedly show a decrease in $\Delta\Psi$ accompanying changes in metabolic states such as the initiation of phosphorylation, or the addition of oligomycin in the presence of ATP (Figs. 6 to 7 and Table 1 of ref. [22]). Furthermore, they show an increase in $\Delta\Psi$ when ATP is introduced to the mitochondrial suspension. Note, however, that in these experiments, Nicholls (see his Materials and Methods, and p. 307, ref. [22]) does not correct for the mitochondrial osmotic decrease in volume following the efflux of K^+ or the increase in volume accompanying addition of ATP. Therefore, the calculated $\Delta\Psi$ would appear to decrease or increase whereas it is likely to remain the same.

The data summarized in Fig. 5 of Nicholls [22] constitute conclusive evidence that the conventional chemiosmotic model does not apply. In that experiment, an electric potential and a ΔpH are maintained with metabolism blocked by rotenone and in the presence of valinomycin and an uncoupler over a wide range of external K^+ concentrations. Valinomycin induces a high permeability to K^+, the uncoupler to protons. The conventional chemiosmotic model predicts a collapse of both ΔpH and $\Delta\Psi$. Furthermore, there is no way in which and electrogenic process can take place in the absence of metabolism. Yet a potential and a ΔpH are maintained. The two cancel out so that the total protonmotive force becomes 0 as might be expected at equilibrium, when $\Delta G=0$ (where G is the Gibbs free energy).

The diffusion potential model proposed here explains the results. The K^+ diffusion potential cannot collapse because the K^+ concentration in the mitochondrial matrix must retain approximately constant to maintain the internal osmotic pressure at a level corresponding to the outside medium (see above). This will be accompanied by mitochondrial volume changes. Equilibrium can only be achieved with a redistribution of the proton gradient so that the two gradients are equal and opposite and correspond to a $\Delta G=0$. The apparent $\Delta\Psi$s in this figure, are somewhat lower than expected because because as explained above Nicholls failed to correct for mitochondrial shrinkage. The results support our arguments:

(1) The presumed $\Delta\Psi$ is purely a function of the external K^+ concentration as expected for a K^+ diffusion potential.
(2) The presumed $\Delta\Psi$ does not require energy.
(3) The internal K^+ concentration is high. In the case of a diffusion potential, the electric potential would be zero when the external and internal K^+ concentration are equal. In the experiment of Fig. 5 of Nicholls, this occurs at a concentration exceeding 70 mM (an extrapolation from Fig. 5 suggests 120 mM, not far from our estimate value for an external K^+ concentration of 150 mM).

Why the contribution of a K^+ diffusion potential in the presence of valinomycin has not been taken into account in many studies is puzzling since the production of a diffusion potential in the absence of metabolism is a well-recognized technique (e.g., [12,26]).

In addition to these considerations, at low K^+ concentration (0.15 mM; ref. [27] p. 432; 0.3 mM,; ref. [23], his Fig. 5), K^+ leaks out of the mitochondria even with metabolism and valinomycin present. These are concentrations at which the presumed $\Delta\Psi$ is high [22]. This behavior is inconsistent with a significant role of membrane potential in K^+ transport in the presence of valinomycin.

If not $\Delta\Psi$, what is responsible for the metabolically dependent uptake of K^+ in the presence of valinomycin? Current information cannot give a definitive answer. The

uptake of K^+ in the presence or absence of valinomycin can be shown to exhibit saturation kinetics when the results of Rottenberg [23] (his Fig. 5) are plotted appropriately on a linear scale rather than a semilog plot as shown in Fig. 3. Furthermore, the addition of valinomycin increases the V_m of the uptake without altering the K_m significantly (K_m=6.1 mM for the control and 4.2 mM in the presence of valinomycin). The results suggest the involvement of a transporter molecule, perhaps a K^+/H^+ antiporter where valinomycin increases its availability or accessibility. The involvement of a K^+/H^+ antiport in mitochondrial K^+ transport is well established (e.g., [28,29]) and has been shown to take place even in the presence of valinomycin. As shown by Massari and Azzone [30], the K^+/H^+ antiport behavior is consistent with the presence of a transporter which becomes more accessible in the presence of valinomycin.

The similar or same Km for the K^+ uptake in the presence and absence of valinomycin cannot be attributed to an indirect effect, for example, an effect on metabolism. In the absence of ADP or uncouplers, the effect of the K^+ concentration on O_2 uptake is minimal (e.g., [31] see Table 1; [32] see Table 3).

2. Experiments using hydrophobic cationic probes

Generally, in mitochondria the $\Delta\Psi$ has been calculated from the concentrations ratios (inside/outside) of cationic probes using the Nernst equation. The hydrophobic cationic probes supposedly have easy access to the mitochondrial inner space. Their distribution has been shown to reflect a K^+ diffusion potential (e.g., 26). To extend the interpretation to measure an electrogenically generated $\Delta\Psi$ is esthetically appealing and the mechanism proposed for the accumulation of the probes has the virtue of simplicity. However, the Nernst equation is derived from the expression of Gibbs free energy. This thermodynamic function is path-independent and the distribution of ions in no way can provide evidence for or against a significant $\Delta\Psi$. The distribution could simply reflect the amount of energy available for the transport and would give the same result if we were dealing with the transport of a non-electrolyte if such a transport system was operative. Expressing the results as voltages is completely arbitrary.

Besides the data from the studies using K^+ and valinomycin which rule out a role of $\Delta\Psi$, there is evidence that the mechanism of the distributions of the hydrophobic probes is not in response to $\Delta\Psi$. The unidirectional influx of triphenylmethylphosphonium ($TPMP^+$) decreases when metabolism is blocked., however, the unidirectional efflux is unaffected (see Table 1, ref. [33]). Such a behavior is not consistent with the presence of a significant metabolically dependent $\Delta\Psi$.

If not a $\Delta\Psi$ what does the distribution of hydrophobic probes represent? If the probes are transported by a non-specific mechanism other then $\Delta\Psi$, perhaps a probe/H^+ antiporter, their distribution would still represent a potential reflecting the energy available to the mitochondria and be treated in exactly the same way as a $\Delta\Psi$, with the understanding that reporting the values as mV is arbitrary and kcal or kJ would be as suitable. For example, the study of Ca^{2+} transport by a uniporter mechanism has been shown to depend on the presumed $\Delta\Psi$ calculated from the tetraphenyl phosphonium$^+$ distribution [34,35]. This finding can be simply re-interpreted based on these considerations.

Leaving the mechanism of the transport of the probes unresolved is not very satisfactory but it is the aim of this presentation to encourage studies that would settle this question as proposed in Section 4 below.

3. Results obtained with microelectrodes

The studies of giant mitochondria using microelectrodes showing no significant metabolically dependent $\Delta\Psi$ have been largely ignored. This is possibly because of the tone of the discussion originally set by Efraim Racker, who likened the insertion of a microelectrode into a giant mitochondrion to “pushing a baseball bat into a cat” [36], ignoring the fact that successful insertions into neurons of similar dimensions had been carried out before. In addition, subsequent work showed that microelectrodes could be introduced into the internal compartment of giant mitochondria (some in the range of 5–10 μm in diameter) with no demonstrable damage, as shown by electrophoretic microinjection of the water-soluble fluorescent dye Lucifer Yellow [37,38] or pyranine [39], which remain in the mitochondria for prolonged periods.

The kinetics of exit of Lucifer Yellow in impaled giant mitochondria support the contention that the microelectrodes do not damage the membrane significantly [38]. The permeability constants (P) calculated assuming a spherical shape are as high as 9.4×10^{-8} cm/s and as low as 3×10^{-9} cm/s. In actual fact the surface of the inner membrane is convoluted (e.g., [40–42]) so that the P values are actually lower (we estimated that surface is approximately 4× greater than that of a sphere) which would be equivalent to permeability constant of approximately 2.4×10^{-8} to 7.5×10^{-10} cm/s. The values reported for the exit of K^+ in human red blood cells are approximately 9×10^{-10} cm/s [43]. The permeability constants for the two systems are not very far apart.

The membrane resistances measured in giant mitochondria with microelectrodes are also in agreement with the contention that there is not significant damage. They were calculated to range from 1 to 4 Ω cm^2 for the giant mitochondria assuming a spherical shape. Correcting for convolutions these values become 4 to 16 Ω cm^2. These values are very low compared to those obtained by Felle et al. in giant *E. coli* [44]. However, they are not far from the

values reported for animal plasma membranes of cells that are perfectly functional such as *Aplysia* neurons (about 20 Ω cm^2) [45], neuroglia (3–10 Ω cm^2) [46], red blood cells (3–10 Ω cm^2) [47], squid axons (25 Ω cm^2) [48] and nodes of Ranvier (8–20 Ω cm^2) [49], although higher values are not infrequent. The last two were obtained with techniques other than the use of microelectrodes and therefore could not result from microelectrode damage. Measurements of conventional mitoplast (mitochondria from which the outer membrane has been removed) patch resistances confirm the values obtained with microelectrodes [50]. They range from 2 to 10 Ω cm^2 assuming a patch 0.5 μm in diameter (the actual size is difficult to determine exactly, see [51,52]). The patch resistances could not be the result of a significant leakage since the patches exhibit normal channel activity. With a significant leak, the current would bypass the membrane.

The impaled mitochondria could still produce ATP at normal rates as shown with either luciferin-luciferase ([53] or a glycerinated myofibril-contraction assay [53,54] (some of the experiments were carried out in the presence of a single giant mitochondrion). An involvement of adenylate kinase can be ruled out by the inhibition of the ATP production by oligomycin or antimycin A. Similarly, Ca^{2+} transport was not significantly impaired by impalement of the giant mitochondria and took place without a significant membrane potential [54,55].

Interestingly, patch-clamping also reveals that even modest electrical potentials across the mitochondrial inner membrane (e.g., −40 mV) induce the opening of the mitochondrial MCCs or megachannels [56] that would collapse any metabolically dependent membrane potential.

4. New experiments

The discussion of the previous sections shows that several questions on the coupling of electron transport to the phosphorylation of ADP or ion transport still require resolution and models alternative to those previously proposed should be examined. Experimental approaches to unresolved questions will be discussed first, followed by a discussion of newer approaches.

4.1. Reexamination of old questions

The mechanism by which hydrophobic cations are transported still remains to be found. The unidirectional kinetics in the presence and absence of metabolism rule out a role of $\Delta\Psi$ [33] (see Section 2). Do these probes use a common mechanism? How is this related to the transport of K^+? Experiments studying the possible competition between probes may be of value. We suspect a non-specific transporter acting as an antiport exchanging cations for H^+ as proposed by Mitchell and Moyle [28] for cations other than the hydrophobic probes.

4.2. New approaches

Experiments with purple membrane [5,7] or lipid layers [6] have shown that free H^+ travel along the surfaces of bilayers (see above). It has been proposed that these events may represent the local effects postulated to have a role in oxidative phosphorylation. Furthermore, as already noted, theoretical considerations [8,9] and experimental results [10] indicate that the coupling of surface protons to a proton sink is entirely possible. Are H^+ generated during electron transport in mitochondria also translocated at the surface of bilayers? Is the surface diffusion of H^+ sufficient to provide protons to the ATP-synthase? Is the location of the produced H^+ sufficiently close to the ATP synthase to permit a direct coupling between the two? pH indicators covalently attached to the outer or inner surface of the inner mitochondrial membrane could provide a test for this model. The indicator could be polar compound that could not permeate the inner membrane or a protein molecule targeted to either surface of the inner membrane through genetic manipulation (e.g., a modified green fluorescent protein). Similarly, the delivery of protons to the surface of the inner mitochondrial membrane directly (e.g. through a patch pipette) or through “a whole cell” type of configuration might be correlated with ATP synthesis. These are feasible approaches.

Mitochondrial matrix pH [57] has been evaluated in intact cells using enhanced yellow fluorescent protein (modified green fluorescent protein) targeted to the mitochondrial matrix and ATP using targeted chimeras of the ATP-dependent photoprotein luciferase [58]. These approaches could be further refined to target specific locations in mitochondria by attaching probes (for example, chimeras of green fluorescent protein or its derivatives) by means of genetic manipulations to mitochondria components such as cytochromes, the ATP-synthase or mitochondrial channels. The production of protons by cytochrome *c* oxidase is very rapid [59]. These approaches should be able to answer the question of whether in mitochondria protons in the bulk phase reach the ATP-synthase rapidly enough to account for the rates of phosphorylation or whether the coupling requires the passage of protons along the surface of the mitochondrial inner membrane.

As discussed above, tomographic reconstruction of mitochondria reveal the possibility of intracristal spaces representing closed or diffusionally restricted vesicles. These spaces could operate analogously to the thylakoid vesicles of chloroplasts when they accumulate high concentrations of protons. The technology to examine this question is relatively simple and can follow the approach of studies of thylakoid vesicles [60,61]. The proposed geometry of the cristae would correspond exactly to that of submitochondrial vesicles which are inside-out in relation to intact mitochondria and are capable of oxidative phosphorylation (e.g., see ref. [62], pp. 145–146).

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